ATTACHMENT A

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Umbilical cord blood-selected CD133$^+$ cells exhibit vasculogenic functionality *in vitro* and *in vivo*

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Abstract

Background aims. Current clinical trials utilize non-selected bone marrow (BM) mononuclear cells (MNC) to augment vasculogenesis within ischemic vascular beds. Recent reports have identified a diminished number and function of hematopoietic stem cells (HSC) from aged and diseased patients. Umbilical cord blood (UCB) provides a potential robust allogeneic source of HSC for therapeutic vasculogenesis. *Methods.* MNC and magnetically isolated CD133$^+$ cells were assessed for viability (trypan blue) and surface phenotype (flow cytometry). To test *in vivo* functionality of the cells, NOD/SCID mice underwent ligation of the right femoral artery followed immediately by cell injection. Blood flow recovery, necrosis, BM engraftment of human cells and histologic capillary density were determined. Cells were tested for potential mechanisms mediating the *in vivo* effects, including migration, cytokine secretion and angiogenic augmentation (Matrigel assays). *Results.* Surface expression analysis showed CD31 (PECAM) expression was greatly increased in UCB CD133$^+$ cells compared with BM MNC. At 28 days, perfusion ratios were highest in animals receiving UCB CD133$^+$ cells, while animals receiving BM CD133$^+$ cells and BM MNC demonstrated perfusion ratios statistically higher than in animals treated with cytokine media alone. Animals receiving CD133$^+$ cells showed a statistically higher capillary density, reduced severe digit necrosis and increased engraftment in the BM than animals treated with unselected BM MNC. *In vitro* studies showed equivalent migration to stromal-derived factor-1 (SDF-1), increased production of tumor necrosis factor alpha (TNF-a) and increased branch points with the co-incubation of CD133$^+$ cells with human umbilical vein endothelial cells (HUVEC) in the Matrigel angiogenesis assay. *Conclusions.* Taken together, UCB CD133$^+$ cells exhibit robust vasculogenic functionality compared with BM MNC in response to ischemia.

Key Words: *adult stem cells, hematopoietic stem cells, transplantation, umbilical cord blood, vasculogenesis*

Introduction

Recent evidence links the level of circulating hematopoietic stem cells (HSC) characterized by expression of CD133 and CD34 with the occurrence of cardiovascular events and death from cardiovascular causes (1–3). This laboratory group and others have demonstrated the efficacy of hematopoietic progenitor cells following short-term culture in restoring blood flow and improving vascular function in animal models of ischemia (4–6). Phase I/II clinical trials have included individual patient-derived autologous bone marrow (BM) culture-derived progenitor cells and whole uncultured BM mononuclear cells (MNC) infused or injected locally in an attempt to augment vasculogenesis in response to ischemia. More recent clinical studies, incorporating further knowledge gained in pre-clinical murine studies, have focused on enriched, non-manipulated stem cell infusions including autologous BM-purified CD133$^+$HSC administered either via intracoronary infusion (7) or intramyocardial injection (8) in patients with coronary artery ischemia. These early pilot studies have reported improved clinical responses, including global left ventricular function and infarct tissue perfusion (7,8).

While the approach of utilizing the patient's own CD133$^+$HSC has the advantage of avoiding potentially adverse inflammatory responses toward

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allogeneic cells by an immune competent host, this approach also has several disadvantages. Patients presenting with acute myocardial infarction may experience significant morbidity with attempted large volume marrow harvest or attempted cytokine-induced marrow stem cell mobilization. Moreover, as the majority of cardiac patients are of advanced age, and increasing evidence points toward reduced regenerative potential (2,9) and decreased responses to inflammatory signals and cytokines released from the ischemic bed (10) as cells age, allogeneic sources could provide adult stem cells with greater capacities. Thus the ready availability of a robust allogeneic HSC cellular infusion without the requirement for extensive *in vitro* culturing may be optimal. Furthermore, because of logistical issues and cost, it is anticipated that cellular therapy may not be widely applicable for the treatment of cardiovascular disease if each individual patient's BM or mobilized peripheral blood stem cells must be collected and processed.

The following study was developed to test the hypothesis that umbilical cord blood (UCB)-derived selected CD133 cells may exhibit an equivalent capability of promoting vasculogenesis in response to ischemia compared with marrow-derived MNC, which is currently used in phase II clinical studies. *In vitro* studies have shown that CD133$^+$ cells migrate to signals elicited by ischemic tissue, and produce cytokines necessary for angiogenesis and promotion of *in vitro* vasculogenesis. CD133$^+$ cells selected from UCB have also been found to be superior to non-selected marrow-derived MNC in mediating neovascularization *in vivo* in the non-obese diabetic/severe combined immunodeficiency disease (NOD/SCID) femoral ligation vascular injury model.

Methods

Cell isolation

Human UCB and adult BM from volunteer healthy donors were collected according to Institutional Review Board (IRB) protocols, including informed consent. MNC were isolated by density-gradient centrifugation from fresh UCB or BM. CD133$^+$ cells were then isolated from MNC using magnetic separation as per the manufacturer's instructions (AutoMACS; Miltenyi Biotec, Auburn, CA, USA).

Assessment of CD133$^+$ endothelial precursor cell (EPC) surface phenotype and purity by flow cytometry

After selection, cells were washed with phosphate-buffered saline (PBS) and counted, and viability determined by trypan blue exclusion. The surface phenotype was evaluated by incubation for 20 min at 4°C with fluorochrome-conjugated monoclonal antibodies (MAb) and appropriate isotype controls: CD133 (Miltenyi Biotec) CD34, CD31 (Becton Dickinson, San Jose, CA, USA), CXCR4, KDR (VEGFR2; R&D, Minneapolis, MN, USA) and CD105 (Endoglin; Serotec, Raleigh, NC, USA). Non-viable cells were excluded based on forward/side scatter and no additional gating was applied. Positive-staining quadrants were set based on non-stained samples and isotype controls. An LSR flow cytometer (Coulter, Miami, FL, USA) was used, acquiring >5000 fluorescence events per sample. Compensation and data analysis were performed using WinList software (Verity Software House Inc., Topsham, ME, USA).

NOD/SCID femoral artery ligation ischemia model

Female NOD/SCID mice (Jackson Laboratories, Bar Harbor, ME, USA) were anesthetized with an intraperitoneal injection of a combination of ketamine and pentobarbital. To further reduce immune-mediated rejection of the injected cells by murine endogenous natural killer cells, the mice were irradiated with a sublethal dose (2.5 Gy) from a Cesium-137 source prior to human cell or control injections. Blood flow of the hind limbs was measured using a laser Doppler imager (moorLDI; Moor Instruments, Axminster, UK) for baseline measurements. Under sterile conditions, a small skin incision was made in the right groin area and the right femoral artery was exposed, ligated along with adjacent branches and transected. The skin incision was then closed with a continuous suture. Blood flow measurements on both feet were repeated 30 min after the femoral artery ligation and each week until the mice were killed (4–6 weeks). All procedures were performed in accordance with the Case Western Reserve University Institutional Animal Care and Use Committee.

After femoral artery ligation, mice were randomized into one of five study groups. Group 1, the control, was treated with an intracardiac injection of EGM2 culture medium (Clonetics, Rockland, ME, USA; 0.3 mL), group 2 with UCB CD133$^+$(0.5×10^6 in 0.3 mL culture medium) and group 3 with BM CD133$^+$(0.5×10^6 in 0.3 mL culture medium). Mice in groups 4 and 5 were injected with non-selected BM or UCB MNC (1×10^6 in 0.3 mL culture medium). Intracardiac injection was performed to avoid first-pass uptake in the lung and liver with intravenous tail vein injection.

NOD/SCID femoral artery ligation ischemia model: necrosis

Following the blood flow measurements, the limb and digits of each mouse were inspected visually

by investigators who did not know the treatment protocol of each mouse. The amount of necrosis was scored on a scale of 0 to 5, with 0 representing no necrosis and 5 representing severe necrosis. The percentage of animals with severe necrosis in each treatment group was then calculated.

NOD/SCID femoral artery ligation ischemia model: histology

At 28 and 42 days after femoral ligation, samples from the lower gastrocnemius in both ischemic and healthy hind limbs were harvested for fresh frozen (liquid nitrogen) and formalin fixation. Neovascularization was assessed by measuring capillary density in fresh frozen sections (40× magnification) of three animals from each study group. Gastrocnemius transverse sections were stained for alkaline phosphatase using indoxyl-tetrazolium and counterstained with eosin to detect capillary endothelial cells, as described previously (11). Two blinded investigators independently counted 20 fields/study animal. Formalin-fixed sections were mounted on saline-coated glass slides and stained with anti-human CD31 antibody (Ab) (Dako, Carpinteria, CA, USA) to identify human cells. Five high-power fields were analyzed independently by two blinded investigators. Sections from control animals were also stained with anti-human CD31 Ab; no positive staining was noted, indicating absence of Ab cross-reactivity with murine endothelial cells. Human tonsil sections were stained as a positive control utilizing the same methodology.

NOD/SCID femoral artery ligation ischemia model: analysis of hematopoietic engraftment of injected cells

Engraftment of injected human cells was assessed by flow cytometry 6 weeks after femoral artery ligation and injection of human cells. Briefly, at time of death BM was isolated and resuspended in Iscove's modified Dulbecco's medium with 10% fetal bovine serum (FBS). The samples were filtered, and red blood cells were lysed following the manufacturer's protocol (lysing buffer; BD Biosciences, San Diego, CA, USA). Cells were incubated for 20 min at 4°C with fluorochrome-conjugated MAb against human CD45. Data were analyzed with WinList (Topsham, ME, USA) software for the percentage human cells in the BM.

In vitro *vasculogenesis analyzes: cell migration assay*

MNC and selected CD133⁺ cells were incubated overnight at 37°C in complete EGM2 media (Clonetics). Cells (50×10^3/150 μL) were added to the upper chambers of Transwells™ (Costar, Corning, NY, USA; 5-μm pores, bare polycarbonate filters) and stromal-derived factor-1 (SDF-1; 100 ng/mL) or vascular endothelial growth factor (VEGF; 50 ng/mL) was added to the bottom chambers. After a transmigration of 3 h, two 250-μL aliquots from the Transwell bottom were used for cell counting by flow cytometry after the addition of 5 μL Fluorosphere™ (BD Biosciences, Franklin Lakes, NJ, USA) beads. Samples were labeled with anti-CD133–phycoerythrin (PE) and anti-CD45-allophycocyanin (APC) Ab to identify CD133⁺ cells.

In vitro *vasculogenesis analyzes: analysis of secreted factors*

To determine the secretion of inflammatory and angiogenic proteins by MNC and CD133⁺ cells from UCB, supernatants were collected from UCB MNC and CD133⁺ cells after 24 h in culture at a cell concentration of 2×10^6 cells/mL. Supernatants were analyzed for inflammatory and angiogenic factors, including basic fibroblast growth factor (bFGF), angiogenin (Ang), VEGF and tumor necrosis factor (TNF)-α, by cytometric bead array kit (BD Biosciences). Standard curves were generated with non-diluted and 1:5 dilution samples and samples were analyzed on an LSR (BD Biosciences) flow cytometer and evaluated using BD CBA software v. 1.1 (BD Biosciences).

In vitro *vasculogenesis analyzes:* in vitro *angiogenesis Matrigel assay*

The Matrigel *in vitro* angiogenesis assay was performed as described previously, with minor changes (12). Passage 2 human umbilical vein endothelial cells (HUVEC) and primary CD133⁺ cells were plated onto 48-well tissue culture plates containing 100 μL growth factor-reduced Matrigel (Becton Dickinson, San Diego, CA, USA) at a density of 2×10^4 cells/mL, with EBM2 medium supplemented with 0.5% FBS. Parallel experiments were conducted in normoxia (21% O_2) and hypoxia (1% O_2) conditions using a PRO-OX chamber (BioSpherix, Redfield, NY, USA). To identify each cell population, HUVEC and CD133⁺ cells were labeled fluorescently with Oregon Green 488 and Vybrant CM-DiI, respectively (Invitrogen Molecular Probes, Carlsbad, CA, USA). Cultures were plated in triplicate and examined after 16 (±1) h under an inverted Nikon 200E fluorescent microscope, for capillary-like structure formation. Photomicrographs of each experimental triplicate were obtained using a 4× objective lens (50–60% well surface area coverage). Two independent, blinded investigators quantified the number of

branch points per high power field. Double-blinded junction counts of the two experimental conditions (HUVEC + CD133$^+$cells and HUVEC + MNC) were compared with the control condition (HUVEC alone). Results shown are mean ± SEM calculated from each triplicate experimental result.

Statistical analysis

Data are summarized by mean ± SEM. Statistical significance was determined between the indicated values with the two-tailed, non-paired, unequal variance Student's *t*-test to test the difference between mean values. A value of $P<0.05$ was considered significant.

Results

Surface phenotype of selected CD133$^+$ cells

Both the starting materials, MNC and isolated CD133$^+$cells, from UCB and BM were enumerated and their surface expression determined utilizing flow cytometry, to evaluate the surface antigen expression of the cells following isolation in comparison with the MNC. The routine yield of CD133$^+$cells from MNC following density-gradient separation was 0.5 ± 0.2% of UCB MNC and 0.7 ± 0.3% of BM MNC, with a post-selection purity of 79.0 ± 2.2% (UCB, $n=30$) and 75.5 ± 4.3% (BM, $n=12$). The surface expression of isolated CD133$^+$cells was compared with BM MNC (Figure 1). Surface expression on sorted UCB CD133$^+$cells was 6.3 ± 2.1% KDR (VEGF-R2), 6.8 ± 3.1% CXCR4 and 20.2 ± 5.5% CD105, compared with 5.5 ± 4.4% KDR, 9.4 ± 1.6% CXCR4 and 14.4 ± 3.6% CD105 in the BM MNC population ($n=3$, expressed as average ± SEM). Notably, the majority of selected UCB CD133$^+$cells (94.8 ± 1.1%, $n=5$) expressed the adhesion protein platelet/endothelial cell adhesion molecule (PECAM or CD31), integral to adherence and transmigration through vascular endothelium (13). Cell viability was maintained after sorting, exceeding 80% (data not shown), prior to use in any assay or *in vivo* injection. These analyzes evaluated the CD133$^+$cells following isolation and prior to usage in other studies.

NOD/SCID femoral artery ligation ischemia model: Doppler blood flow

MNC and CD133$^+$cells from UCB and BM were utilized in a NOD/SCID femoral artery ligation ischemia model to investigate their ability to augment neovascularization in an *in vivo* ischemia model. Figure 2A shows representative images from mice at baseline (prior to femoral ligation), immediately post-ligation and 28 days following injury and injection of human cells. Selected CD133$^+$cells from UCB provided at a lower dosage (0.5×10^6) were noted to be more potent in

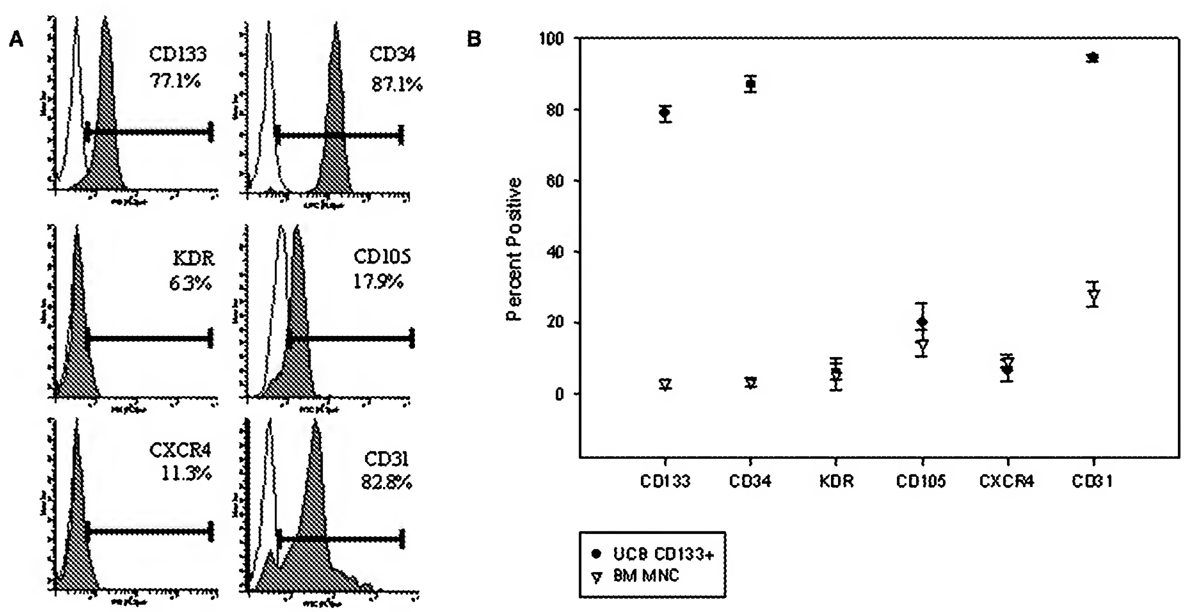


Figure 1. UCB CD133$^+$cells have high purity and exhibit expression of CD34, KDR, CD31, CD105 and CXCR4. MNC were isolated from UCB and BM with density-gradient centrifugation. CD133$^+$cells were isolated by magnetic separation (Miltenyi). Surface expression was evaluated with fluorochrome-conjugated MAb and appropriate isotype controls and >5000 fluorescence events were acquired per sample. Open histograms represent negative controls. (A) Representative histograms from one UCB CD133$^+$sample; (B) average ± SEM ($n\geq3$).

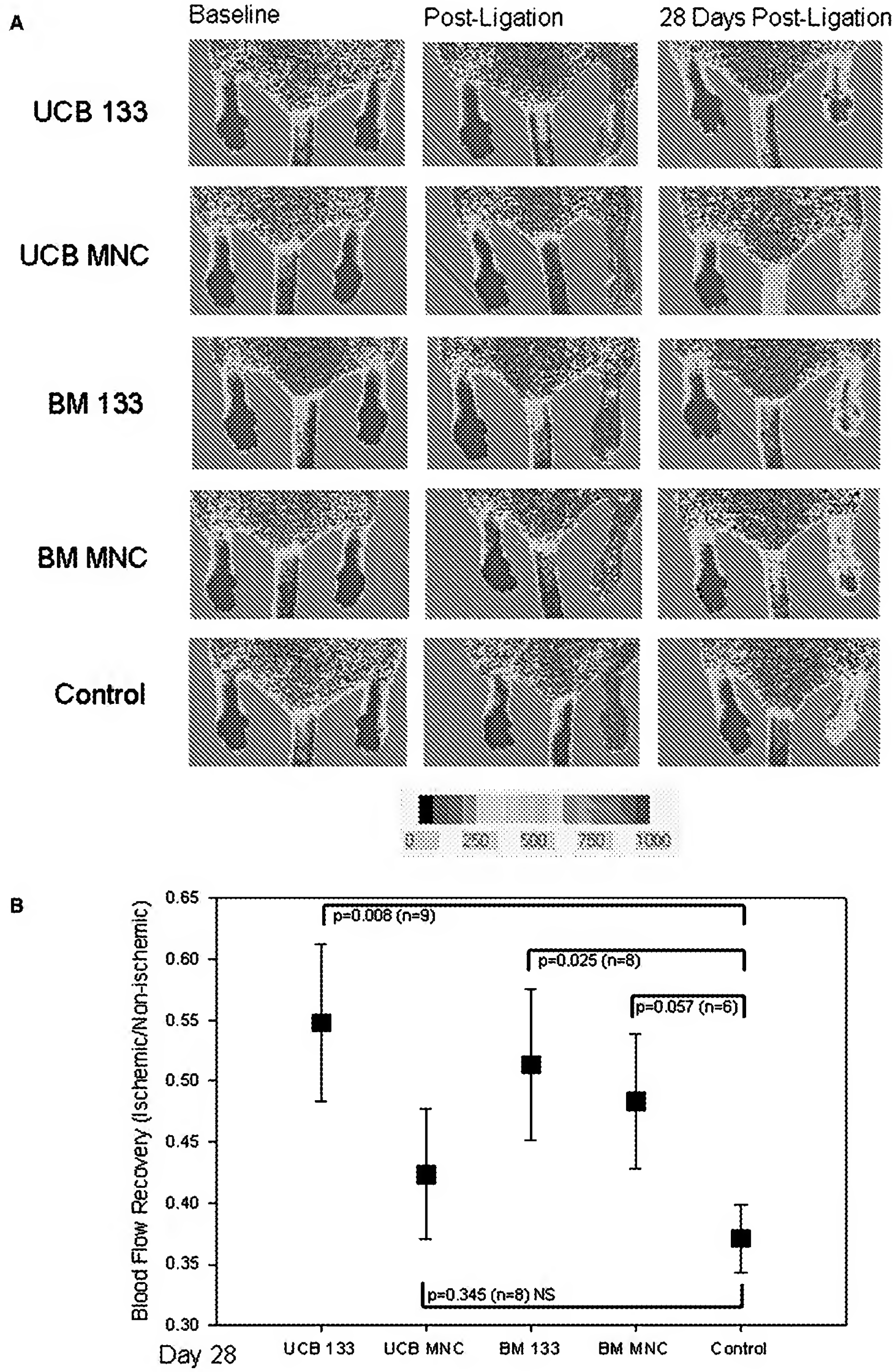


Figure 2. CD133$^+$HSC increase blood flow recovery ratios after injection into NOD/SCID mice with induced hind limb ischemia. Blood flow recovery ratios after injection of MNC or CD133$^+$cells into NOD/SCID mice with induced hind limb ischemia. Control animals were injected with complete EGM2 media. Doppler measurements were taken immediately after ligation and then on days 7, 14, 21 and 28. Perfusion ratios were determined between the ischemic and non-ischemic leg of each study animal. Values shown are average $\pm$SEM, UCB CD133$^+$(n=9), UCB MNC (n=8), BM CD133$^+$(n=8), BM MNC (n=6), Control (n=12).

mediating improved blood flow compared with a larger dose (1.0×10^6) of non-selected BM MNC, the cell populations used in current clinical trials. At 28 days (Figure 2B), perfusion ratios were highest in animals receiving UCB CD133$^+$cells (0.55 ± 0.06, n=9), while animals receiving BM CD133$^+$cells (0.47 ± 0.07, n=8) and BM MNC (0.48 ± 0.8, n=6) demonstrated perfusion ratios statistically

higher than those measured in animals treated with cytokine media alone (0.37 ± 0.03, $n=12$, $P<0.05$). Comparison between blood perfusion ratios after injection of CD133$^+$ cells derived from UCB and BM was not significant ($P=0.71$).

NOD/SCID femoral artery ligation ischemia model: histology

Tissue samples of femoral muscle distal to arterial ligation were evaluated for capillary density as a possible histologic mechanism of the observed increased blood flow. Representative photomicrographs are shown in Figure 3A. Control animals demonstrated a density of 131 ± 6.9 cells/mm^2. As seen in Figure 3B, capillary density was statistically higher than controls in animals receiving UCB CD133$^+$ (320 ± 18; $P<0.0001$), BM CD133$^+$ (183 ± 9.3; $P<0.0001$) and UCB MNC (164 ± 10.5; $P=0.011$). Mice treated with BM MNC (135 ± 9.4) did not have a statistically significant increase in capillary density from controls ($P=0.73$). In addition, animals treated with both UCB and BM CD133$^+$ cells had statistically higher capillary density than unselected MNC ($P=<0.0001$ and $P=0.0004$, respectively).

Histologic samples from day 28 and day 42 were stained for human CD31 (huCD31) in an attempt to locate human cells embedded in the murine ischemic tissue. Our analysis found no positive staining for huCD31 in animals in any treatment arm (data not shown). Human tonsil was used as a positive control and showed characteristic staining for huCD31.

NOD/SCID femoral artery ligation ischemia model: necrosis

At each blood flow measurement, mouse limbs and digits were evaluated and scored for necrosis on a scale from 0 (no necrosis) to 5 (severe necrosis). This measurement was used as a physical evaluation in addition to blood flow measurements. The percentage of animals with severe necrosis in each study group was then calculated. Only 10% of animals receiving UCB CD133$^+$ cells showed severe necrosis, whereas in the control group 69% of animals showed severe necrosis (Figure 3C). Examples of animals scored without necrosis and with severe necrosis are shown in Figure 3D.

In vitro *cell migration*

We measured chemotactic migration of CD133$^+$ cells and UCB MNC towards SDF-1 (100 ng/mL) and VEGF (50 ng/mL) chemotactic agents, compared with control wells containing media alone (Figure 4A). The fold increase over control of migration to SDF-1 was 1.8 ± 0.08 UCB MNC and 1.7 ± 0.08 UCB CD133$^+$ ($n=3$). When migrating to wells that contained VEGF, UCB MNC had a 1.6 ± 0.2-fold increase over wells without any stimulus and UCB CD133$^+$ cells showed a 1.1 ± 0.09-fold increase. These studies indicated that selected CD133$^+$ cells retained expression of the homing receptors and retained a functional response to the chemotactic ligands SDF-1 and VEGF.

Cytokine production

Supernatants generated from UCB CD133$^+$ and UCB MNC showed production of TNF-α, VEGF, Ang, bFGF and interleukin (IL)-8. Results shown in Figure 4B demonstrate statistically significant elevated levels of TNF-α secretion in UCB CD133$^+$ supernatant (81.3 pg/mL) compared with UCB MNC supernatants (12.0 pg/mL), with $P=0.009$. IL-8 and bFGF produced by CD133$^+$ and MNC from UCB were equivalent. Production of VEGF and Ang was elevated in UCB MNC compared with UCB CD133$^+$ ($P=0.02$ and $P=0.01$, respectively).

In vitro *angiogenesis assay*

In order to determine the effect of the addition of UCB CD133$^+$ cells or UCB MNC on the formation of capillary-like structures, a Matrigel *in vitro* angiogenesis assay was used. All assays were done under normoxic and hypoxic conditions. HUVEC were plated alone as a control and UCB CD133$^+$ cells or MNC were added to the experimental wells. The addition of CD133$^+$ cells produced a significantly greater number of branch points in normoxic conditions (13.2 ± 1.5) compared with MNC (2.5 ± 0.45, $P=0.0009$, data not shown). As shown quantitatively in Figure 4C and qualitatively in Figure 4D, under hypoxic conditions the number of branch points was increased further in cultures incorporating UCB CD133$^+$ cells (19.4 ± 2.4) compared with MNC (9.5 ± 0.84, $P=0.0003$). Figure 4E illustrates the capillary-like structures formed by the HUVEC (green) when CD133$^+$ cells (red) were added to the assay. CD133$^+$ cells were noted to associate within the branch points ($n=3$).

NOD/SCID femoral artery ligation ischemia model: hematopoietic engraftment

In addition to blood flow recovery and classic histology in mice injected with cells, we analyzed marrow engraftment of human HSC *in vivo*. At the time of killing, BM was harvested to assess engraftment of human cells by flow cytometric analysis. Figure 5A shows quantitatively that mice injected with UCB CD133$^+$ cells showed higher expression, with 19 ± 4.9% positive huCD45 cells compared with 2.5 ± 0.6% for UCB MNC, 1.6 ± 0.4% for BM CD133$^+$ cells and 2.3 ± 0.3% for BM MNC ($n=3$). Representative histograms are shown in Figure 5B.

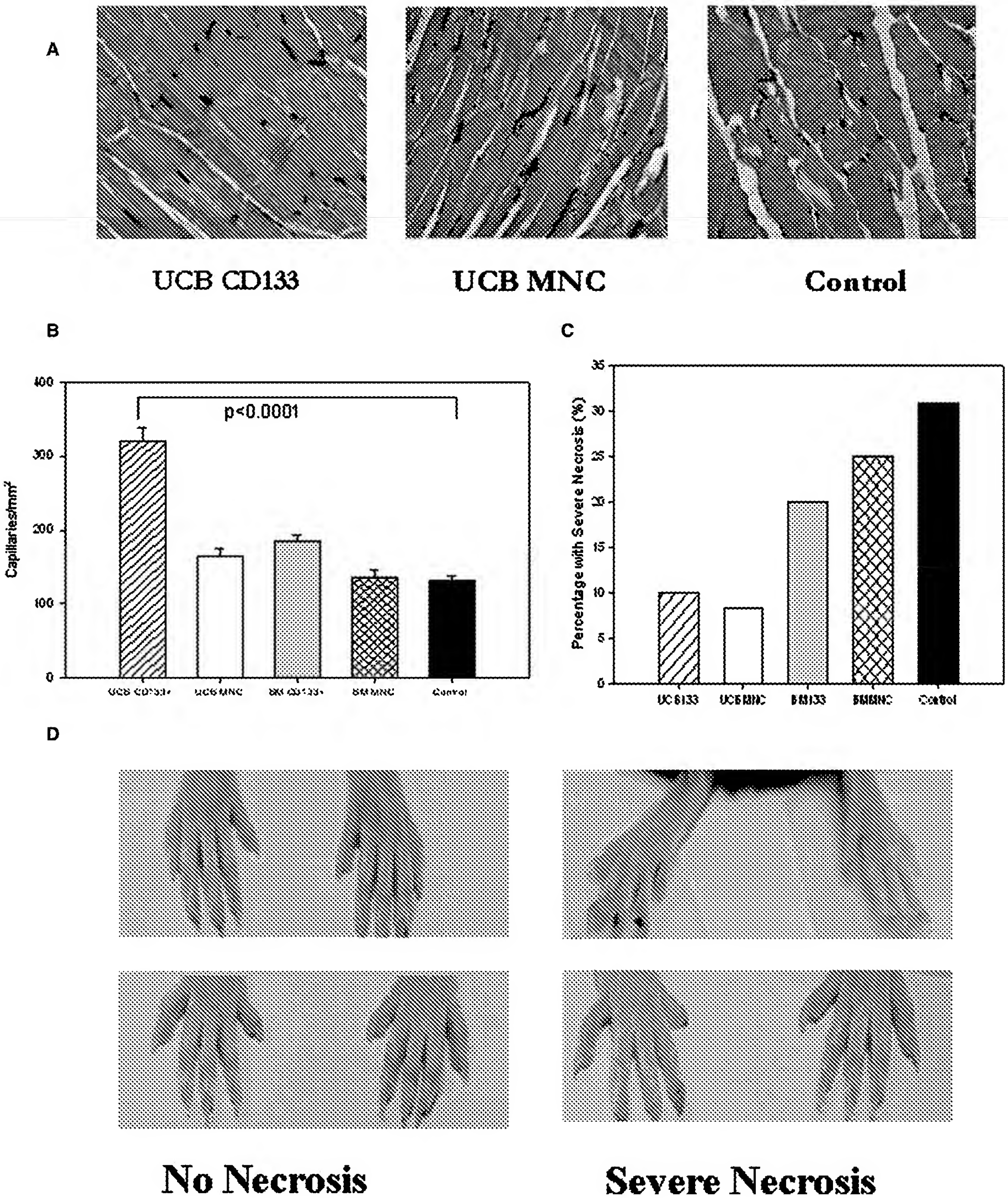
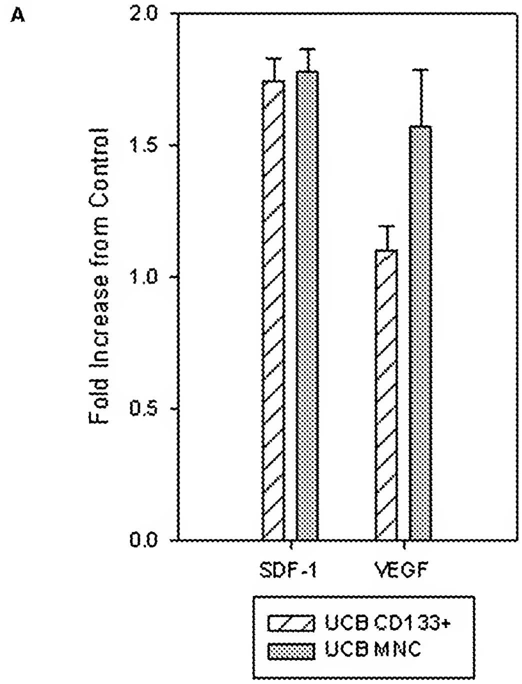


Figure 3. Injection of UCB CD133$^+$cells into mice with induced ischemia increases capillary density and reduces severity of necrosis compared with other cell types. At 42 days after ligation of the right femoral artery, tissue was harvested from the lower calf muscle of study and control mice. Two blinded investigators counted 20 fields per sample and capillary density was expressed as capillaries/mm^2. Representative photomicrographs of histologic sections used to determine capillary density are shown in (A). (B) Injection of UCB CD133$^+$cells increased the capillary density compared with all other treatments ($P<0.001$), with $n=3$ in each group. During each blood flow measurement the ischemic limbs were scored on a scale of 0–5 for severity of necrosis, with 0 representing no visible necrosis and 5 representing the most severe ($n\geq 6$ for each group). (C) Results of percentage distribution of mice that exhibited severe (4–5) necrosis. Mice receiving UCB CD133$^+$cells showed a lower incidence of severe necrosis, with the majority of mice having mild necrosis. (D) Representative animals scored with no visible necrosis and with severe necrosis. Photomicrographs were taken at 20×for all samples.



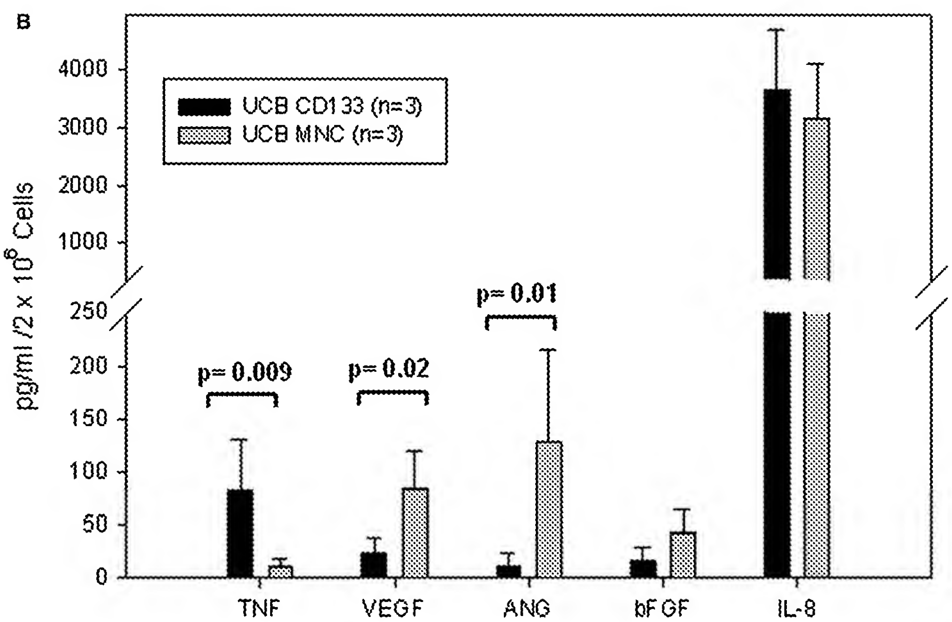


Figure 4. CD133$^+$cells from UCB demonstrate *in vitro vasculogenic* capacity as examined by migration, cytokine production and Matrigel. Migration to angiogenic factors SDF-1 and VEGF is shown in (A). Gray bars represent the fold change of UCB MNC cells in wells with VEGF and SDF-1 over those wells with no cytokine present. The striped bars represent fold change of UCB CD133$^+$cells in wells containing VEGF and SDF-1 over those wells without cytokines. The cytometric bead assay was utilized to detect cytokine production from UCB CD133$^+$and MNC after 24-h culture (B). Quantification of branch points or capillary-like structures of CD133$^+$and MNC from UCB compared with HUVEC alone are shown in (C); (D) shows the increased number of HUVEC (green) with the addition of CD133$^+$cells. CM-Dil-labeled CD133$^+$cells associate with developing HUVEC tubules in Matrigel after 24 h and increase the number of branch points/capillary-like structures, as shown in (E).

Discussion

Autologous marrow-derived hematopoietic stem and progenitor cell therapy has been tested in pre-clinical studies and clinical phase I/II trials, with infusion or local injection, in an attempt to improve neovascularization and heart function after ischemia. The potential benefit, if any, in the use of autologous cellular therapy for patients with ischemic cardiovascular disease has been addressed in recent studies, in which Schachinger *et al.* (14), Assmus *et al.* (15)

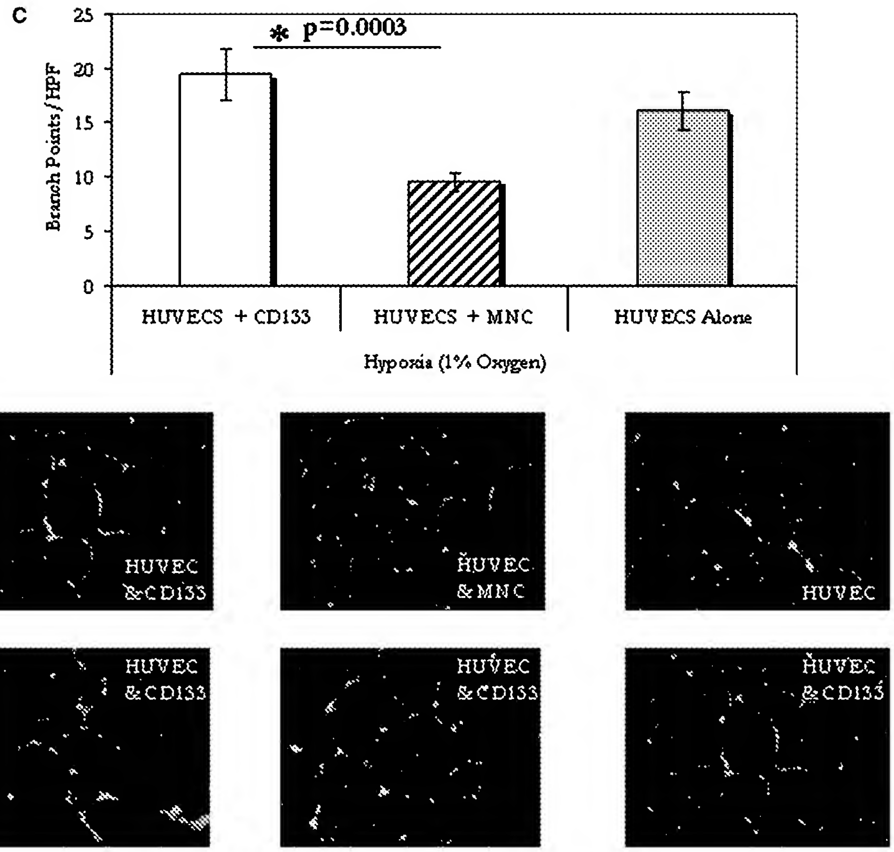


Figure 4. (*Continued*).

and Lunde *et al.* (16) report conflicting data in randomized phase II trials, providing a cautiously optimistic perspective on this approach and indicating the important need for further basic and translational research to guide clinical investigation.

Taken together, these early clinical studies reported variable efficacy and did not control for specified cell populations infused with therapeutic intent, nor did they address targeted cell doses for infusion. In the phase II double-blind REPAIR-AMI trial, those acute myocardial infarction (AMI) patients receiving non-selected intracoronary MNC infusions containing a larger absolute number of marrow CD34^{+}CD133^{+} cells (median 1.8×10^{6}), infused 3–5 days after successful reperfusion therapy, demonstrated significantly improved left ventricular ejection fraction at 4 months follow-up compared with AMI patients not receiving intracoronary cell infusions (14). In comparison, in a second, smaller, randomized study (16) AMI patients received a lower absolute number of marrow CD34^{+} cells (median 0.7×10^{6}) infused at a median of 6 days after reperfusion therapy. No improvement in global left ventricular function was noted. These variable clinical trial data underscore the importance of identifying the specific cellular constituents underlying observed beneficial effects, and the need to test specified cell doses to gain further understanding of the mechanisms underlying therapeutic stem cell-mediated augmentation of microvascular vasculogenesis in response to ischemia.

Impairment of the number and function of patient-derived HSC and progenitor cells has been confirmed by several groups and is thought to limit the efficiency of autologous stem cell therapy (2,17–19). An optimal cell product for clinical use includes robust cell proliferation and cytokine production, ease of procurement and ready access, without the requirement for extensive *in vitro* culturing prior to infusion. Analyzes reported herein show UCB-derived CD133^{+} cells demonstrate equivalent enhanced vasculogenic functionality compared with standard marrow-derived MNC currently tested in human clinical studies. CD133-enriched cells from marrow and UCB exhibited equivalent surface markers, including VEGFR2 (KDR), CXCR4 and CD31 (PECAM), allowing equivalent chemotaxis to chemokines elicited in ischemic tissues, including SDF-1, and the ability to adhere and extravasate in hypoxic vascular regions. In addition, CD133^{+} cells selected from UCB MNC secreted robust quantities of cytokines supporting angiogenesis, including TNF-α, VEGF, bFGF and Ang.

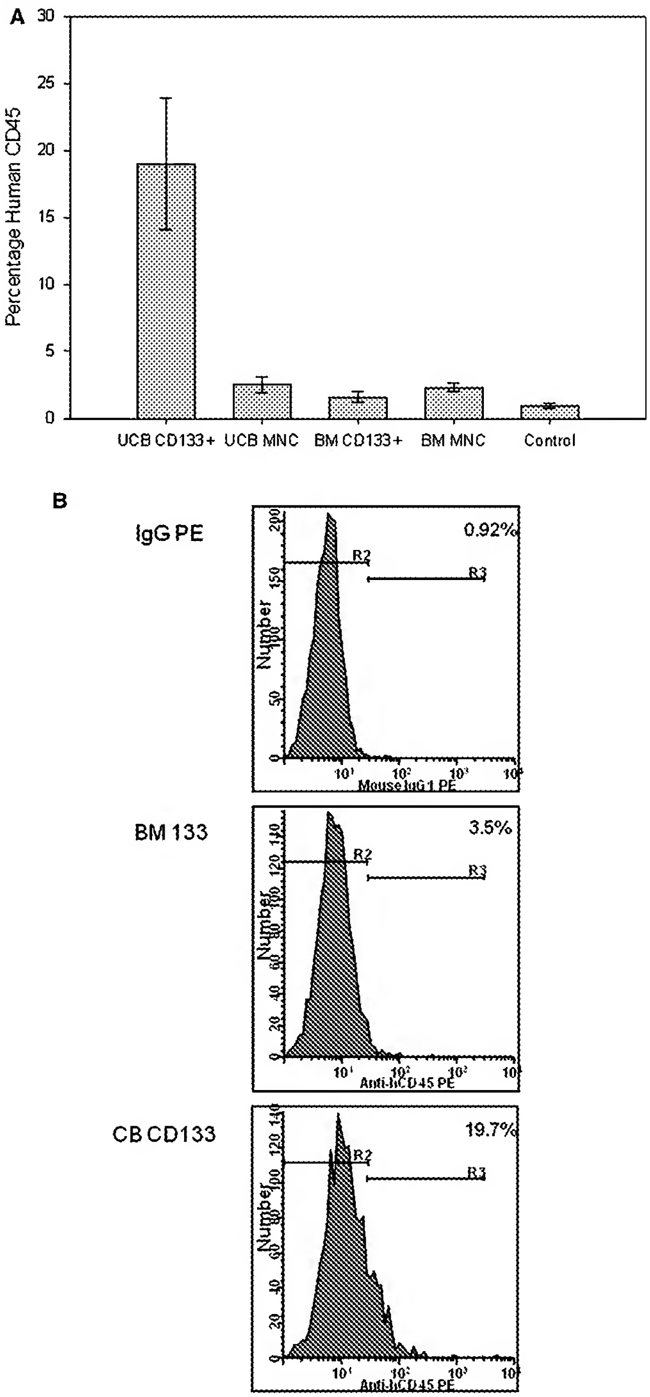


Figure 5. BM engraftment as measured by huCD45 in mice treated with human cells following induced hind limb ischemia. Subsets of animals were kept alive for 42 days in order to measure BM engraftment. Flow cytometry for huCD45 was used to measure engraftment in three mice in each treatment group (A). (B) Representative histograms of isotype controls and experimental samples of mice injected with UCB 133^+ and BM 133^+ cells.

The addition of $CD133^+$ cells to HUVEC in Matrigel assays produced more branch points (i.e. more vessels/capillaries) than the addition of MNC and, interestingly, $CD133^+$ cells were noted to localize to new branch points formed by HUVEC.

Taken together, $CD133^+$ cells selected from UCB exhibited robust vasculogenic functionality compared with BM MNC in response to ischemia. Although the vasculogenic capability of UCB-derived progenitors has been described previously (20–22), to date no direct comparative studies with marrow-derived progenitors has been performed. NOD/SCID injected with human cells in this study, including non-selected MNC as well as enriched $CD133^+$ cells from UCB and marrow, demonstrated improved blood flow after femoral ligation over that of cytokine media controls. This may reflect, in part, xenotransplant immune responses supportive of the vasculogenesis response, as this immunodeficient murine model, although lacking adaptive T- and B-cell immune responses that thereby allow the engraftment of human cells, retains innate immune responsiveness known to participate in early vasculogenic responses (23,24).

Interestingly, UCB-derived $CD133^+$ cells, compared with UCB-derived MNC, secreted significantly more TNF-α, suggesting a relationship between increased TNF-α secretion by $CD133^+$ cells and their improved vasculogenesis. Of note, contemporary clinical trials of TNF inhibitors (25,26) in patients with congestive heart failure and ischemic heart disease showed no benefit, and possibly harm, in patients receiving TNF inhibitors. These clinical study results combined with our current findings suggest a necessary role of TNF-α in cell-based vasculogenesis, providing a possible mechanistic explanation for the equivocal and, possibly, deleterious effects of TNF-α inhibition in clinical trials for heart failure patients, the majority of whom (greater than 50–60% in most trials) have underlying coronary artery disease.

An alternative explanation is that some level of basal TNF-α activity is necessary for successful and effective vasculogenesis. The significantly higher TNF-α cytokine secretion from $CD133^+$ cells selected from UCB, compared with that from unselected UCB MNC, may provide one underlying mechanism explaining the vasculogenic advantage of $CD133^+$-selected cells over unselected MNC. This hypothesis also suggests a promising role for allogeneic $CD133^+$ cells selected from UCB in vasculogenesis in patients qualifying for cell therapy for refractory ischemic heart disease, for example. While speculative, these hypotheses warrant further laboratory and clinical investigation.

Of note, blood flow in NOD/SCID mice 4 weeks after femoral ligation was significantly higher in animals injected with UCB-derived $CD133^+$ cells despite administration of a lower cell dose (0.5×10^6 $CD133^+$ versus 1.0×10^6 MNC), and histologic analyzes confirmed improved measured Doppler

blood flow. There was no significant difference between UCB MNC or BM MNC and control at 28 days (P>0.05 in both cases). Not surprisingly, $CD133^+$from BM significantly increased blood flow at day 28 compared with controls, highlighting the benefit of using a selected cell population instead of heterogeneous MNC. In addition, examination of human cell engraftment in the marrow was highest in NOD/SCID receiving cord blood-derived selected $CD133^+$cells. It is not clear whether the effects mediated by the injected human cells were primarily paracrine or direct cell–cell cyoprotection; however, improved microvascular vessel density was totally murine-derived, and human cells were not observed to incorporate anatomically in murine vasculature post injury and cell injection. Sustained engraftment of UCB $CD133^+$cells in the murine marrow is noteworthy, rendering this NOD/SCID study model possibly ideal for future evaluation of specific inflammatory and angiogenic cytokines and/or circulating marrow-derived cells supporting murine-derived vasculogenesis within the distant ischemic vascular region.

Whether injected HSC facilitate neovascularization by direct cellular interactions and/or secondary paracrine effects has not been well delineated (27). Evaluation of these interactions does not always yield exact correlative results. We found that the blood flow measurements were not solely defined by one *in vitro* property. Therefore, we investigated surface expression, migratory capacity and secretion of angiogenic factors in an attempt to delineate a broader spectrum of influence. Serum factors including VEGF and TGF-β1 have been shown to augment endothelial cell proliferation (28). The observed functional improvement measured by Doppler blood flow and physical assessment of necrosis, as well as histologic evidence of improved regional perfusion exerted by UCB $CD133^+$cells without anatomic incorporation, supports the concept that post-injury augmentation of murine-derived microvascular vasculogenesis is primarily paracrine-mediated in animals treated with human $CD133^+$cells.

Although concern is raised regarding potential adverse immunologic responses to administered allogeneic $CD133^+$HSC, this risk is weighed against the use of a robust UCB-derived cell population with respect to proliferation, homing and cytokine secretion under hypoxic conditions compared with that of $CD133^+$HSC derived from aged patients with cardiovascular disease. Further, potential adverse innate immune responses could be regulated by a specified low cell dose of allogeneic cells infused or injected with intent to stimulate innate immune responses in a measured fashion to augment vasculogenic responses within the ischemic bed. Taken together, the complexity underlying hypoxic vascular endothelial signaling mediating homing and participation of allogeneic $CD133^+$HSC warrants further systematic evaluation of these purified cell populations at specified cell doses, in order to gain an understanding of the relative role of HSC in vasculogenesis responses within an ischemic bed.

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